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Differences in signalling by directly and indirectly binding ligands in bacterial chemotaxis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 April 2010

Thank you very much for submitting your paper for consideration to The EMBO Journal editorial office.
As you will see from the enclosed reports two referee's are very supportive of your study, whereas ref#2 concludes that at least the current version of the manuscript would rather be more suited for more specialized audience. Careful reading of all the reports than reveals that necessary experimental extension and a complete rewrite could make the study accessible and thus suitable for the more general readership of our journal. More specifically, experiments to investigate responses to complex mixtures of effectors as suggested by ref#2 (and similarly eluted to by ref#3) and putting those into the context of other signaling strategies should significantly improve the general impact of the study and would thus be essential to complement the current results.
As the referee reports do provide otherwise ample and constructive suggestions how to improve the paper, there is not much need for me to repeat all their points here in detail. All in all, I am very happy to offer you the possibility to revise the study accordingly. I also have to remind you that it is EMBO_J policy to allow a single round of major revisions only. Thus, the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript that will be assessed from some of the original referees!

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is a beautiful study that provides important new information about chemosensory responses mediated through periplasmic binding proteins. The data are compelling and the modeling analysis nicely complements and explains the experimental findings

The quantitative treatment relies on accurate measurements of the relative numbers of various types of receptors in the cells. This report finds rather different values than a previous study by Li & Hazelbauer, which has been treated as gospel in the literature. I believe the new numbers, but suggest that the authors expend a few additional sentences to explain how they used fluorescently-tagged receptors to calibrate the western blots (if I understand the method used) and why their measurements differ from those of Li & Hazelbauer.

Otherwise, the presentation is nearly flawless. I can only offer minor suggestions for improving clarity in spots.

p.2, lines 5-7: delete "directly", change "to demonstrate" to "and found" and delete comma after "new".

p.3, line 4: change "is" to "are"

p.3, line line 6: delete "stable"

p.3, lines 11-12: add "to an attractant stimulus" before "works"; change "to attractants" to "the attractant".

p.3, line 14: delete "clearly"

p.3, line 16: change "the first" to "one"; change "The second" to "a second".

p.4, line 9: change "inside" to "within".

p.4, line 21: add "the" before "corresponding"; line 23: change "is" to "are".

p.5, lines 9-10: I wouldn't call these EC50 values "comparable". They seem to range over several logs.

p.7, line 12: change "that" to "than".

p.13, line 11: Perhaps add comma after "unsaturated".

p.16, line 6: "microscope" is misspelled.

Referee #2 (Remarks to the Author):

The manuscript by Neumann, et al. seeks to compare chemotaxis signals derived from componds that bind directly to receptors versus those that act through periplasmic binding proteins (BPs). Ligands were evaluated for their ability to elicit changes in CheA activity by using FRET between CheY-YFP and CheZ-CFP, a technique previously used by the Sourjik lab. The main conclusion from these studies is that indirectly binding ligands have a smaller dynamic range of concentrations that will illicit signaling responses. The authors also updated a mathematical description of chemotactic signaling to account for indirectly binding ligands. These observations led the authors to update our current understanding of chemotactic signaling by suggesting that the binding mode of effectors contributes more to chemotaxis response than population differences of the chemoreceptors that mediate signaling with these effectors.

This manuscript is uncharacteristic of those from the Sourjik lab, which typically provide compelling evidence for their conclusions. As presented, the manuscript would be unlikely to be interesting to a broad audience and therefore appears to be better suited for a more specialized journal. Though interesting to those focused on bacterial chemotaxis, the findings presented here are likely to be of interest to the group of researchers focused solely on bacterial chemotaxis.. If the current study were extended to investigate or predict chemotactic response to complex mixtures of effectors, the impact of the current study would be greater. Alternatively, a discussion of how the authors' observations impact bacteria in physiological settings or their ramifications for other signaling pathways also would raise the impact of the paper.

General comments:

1. The manuscript is a very tough read in that is written bacterial chemotaxis insiders. It is difficult to discern the motivations behind the executed experiments. Context is missing also in that a discussion of reported prior experiments is needed.
2. While the FRET technique utilized here has been developed and used in previous reports, a brief overview of how the FRET experiments were conducted (in the main text) is needed so that readers can evaluate what the responses generated represent at a molecular level.

a. The authors do not address in this manuscript whether the dynamic range of signaling observed is limited by the chemotaxis sensory system or by limits of the FRET system itself.

b. How do the authors rule out that the signaling differences that they observe for the periplasmic binding proteins are not due to competing cellular uptake and metabolism of various signaling molecules? What is the affinity of the transporter versus the chemoreceptor for the BP-signal complex? How does BP uptake compare with internalization via symporters?

3. The authors quantified the amount of each receptor and used these amounts to normalize effector response. This analysis could be altered by processes noted in 2b above.
4. It was noted that the ratios of direct and indirect signaling receptors in this study differ from those previous reported, and this is a significant motivation for their proposed model in the Discussion. However, the authors did not cite those previous studies in the text. Moreover, what is lacking is an interpretation for the discrepancy in the quantity of receptors in this and previous studies. This ambiguity could lead readers to question which data is more reliable. Indeed, are the levels of receptors influenced by the growth conditions? If so, what levels are physiologically relevant?
5. The authors did not provide context to previous experiments that investigated the dynamic range of receptor response in the Results section. It appears that the values obtained in this study agree with those reported in the 1970s. It would be appropriate for the authors to discuss these previous experiments at the outset of the study. This would also provide more context to the importance of their findings with indirect binding ligands.
6. It was unclear how the mathematical model augments this study. There was no context given in the Results section and little, if any, discussion provided. The authors should delineate what insights were gained from this updated model. Could they test their model with new experiments? Along these lines, the data presented in Figure 5 were not evaluated. It was unclear what this data provided to the other findings described.
7. One of the main conclusions is that the smaller dynamic range of indirect ligand binding is not due to inefficient adaption, but due to the requirement for accessory binding to BPs. The authors do not provide sufficient experimental evidence to support their model.
8. Standard models for signaling output predict that processes that involve multiple proteins coming together will occur with a higher level of cooperativity and therefore one would predict periplasmic BPs should result in responses over a narrow range of concentrations.

8. Minor Comments. Several issues are unclear in the text as noted below.

a. Figure 4C - is the WT panel mislabeled, or is the description provided incorrect? If the description is correct, the WT cells should provide the lowest response to effector.

b. The last sentence on page 11 in the discussion was unclear. Most of this seems to be due to imprecise wording. It is unclear what the authors mean by "non-optimal binding properties of the BP" in regards to galactose signaling.

c. The sentence on page 12 referring to Koff and Kon is confusing. Are the authors referring to rate constants (typically lower case k) or are these equilibrium constants for the on and off states? If it is the latter, the nomenclature is not parallel to that used earlier.

d. The use of the term "saturation" with regard to the receptors is confusing. Saturation with receptor ligand interactions has a very different meaning so I found the use of "saturating" levels of

receptor to be confusing.

Referee #3 (Remarks to the Author):

This is a monumental study, impressive for its thoroughness both in experiment and modeling. It has one major drawback, however, which is that it is relatively hard to read. I feel that the authors have failed to make the best case that they can for the importance of their results. The paper should be rewritten to increase its clarity and accessibility.

To me, the single most important conclusion from this work is that chemotaxis is matched to different mechanisms of transport. For ABC transporters that utilize periplasmic binding proteins, both chemotaxis and transport share a binding protein as a common element. In such systems, the membrane components of the system are present at much lower levels (50-100 fold) than the binding proteins after full induction of the systems. Therefore, cells can bind far more ligand in their periplasm than they can transport. There is little point in swimming to a concentration of an attractant that is near or above the Vmax for transport, which is relatively low for such systems.

Transport does, however, significantly diminish the level of substrate available in the periplasm at moderate concentrations of maltose - and presumably other binding-protein substrates - which has the effect of broadening the dynamic range for chemotaxis and shifting it toward higher concentrations. For example, cells that are defective for maltose transport show peak accumulations in capillary assays at tenfold lower concentrations of maltose than cells with intact transport systems (see Zhang et al., JBC 1999, and references therein).

Some of these points are made in the Discussion of the current manuscript, but almost as an afterthought. I think they should be front and center. A title that would embody this idea would be something like "Correlation of mechanisms for chemotaxis signaling and substrate transport in Escherichia coli." If the authors insist on focusing on chemoreceptor function, they should still emphasize the difference, as in a title like "Chemoreceptors signal differently in response to ligands that bind directly and indirectly." In any case, the Abstract should be rewritten to highlight the really interesting, rather than the more arcane, inferences from the work.

Being a molecular biologist with limited math skills, I am in no position to comment on the modeling aspects of the paper. I hope that another reviewer can do that. I am impressed that the modeling and experimental results either match or differ in ways that can be plausibly explained.

The main question that I have about the experimental results is why aspartate was never tested. It is the direct-binding attractant with the lowest EC50. and it would seem to be important to include aspartate to make the comparison complete, since serine and its non-metabolizable, chemotactically less-potent analog AIBU were both tested. I realize that, at high concentrations, it may also be sensed by Tsr, but that should interfere too much with the measurements at micromolar concentrations.

That brings up one other concern, which is the invocation of a mysterious binding protein that could mediate a Tsr-independent response to serine. Why couldn't that just be a low-level response to serine mediated by Tar? This hypothetical binding protein, whose existence is based on circular logic, i.e., a marginally smaller dynamic range, should be eliminated unless there is firmer evidence for its existence.

I have a number of minor comments, in addition to many suggestions for changes in wording and punctuation, which can wait for a later time:
1) Citations clustered in the text should be in chronological order.
2) Macnab and Koshland should also be given credit to temporal comparisons of chemoeffector concentrations.
3) Hazelbauer (1975) was the first to show that MBP is the primary sensor of maltose, and Manson and Kossmann (JB, 1986), not Manson et al. (1985) were the first to give solid genetic evidence for the direct interaction of MBP and Tar.
4) On the first page of Results, the statement that all ligands showed comparable EC50 values is very misleading and in direct contradiction to the date shown in Figure 1.

5) On the same page, it should be briefly explained here in what strains and how binding protein were overexpressed.
6) Is there a PTS for maltose? Isn't the response likely to be to glucose contamination? Otherwise, why would cells lacking the ABC transporter for maltose be unable to grow on even high concentrations of pure maltose?
7) Page three of results. It should be explained briefly in the text how the immunoblots were quantified. Also, in the quantification of chemoreceptors in strains W3110 and RP437 (Fig. S3D and E) really comparable, and even if so, how do the authors explain the very different results of Li and Hazelbauer (2004)?
8) Page four of Results. No data are given for Tar in Figure 5SD, as the text implies.
9) Same page, what is "the total receptor level?" Is this relative to TSR with a value of 1? Not clear.
10) Top of next page. When the authors say the Kon and Koff are binding constants for a particular type of receptor, does that mean a particular receptor in a particular methylation state? Because methylation can change ligand affinities (an unexplored topic for indirectly binding ligands, as far as I know), it is important to be very clear about this.
11) First page of Discussion, bottom line. What is meant by "the non-optimal binding properties of the BP?"
12) Break up the run-on paragraphs in the Discussion into more digestible segments.
13) It seems odd to say (see text for details) in the Materials and Methods.
14) Bottom of the first page of Material and Methods, the sentence beginning here and continuing to the next page is long and tortuous.
15) Second page of Material and Methods. Is "480/40 nm band pass" correct, and if so, what does it mean? +/- 40 nm, 460-500 nm?
16) Could the reason that the titration curves with galactose, ribose and Pro-Leu do not approach zero kinase activity be that some receptor teams do not contain Trg or Tap. (That would seem more likely with Li and Hazelbauer's receptor ratios.)
17) I realize that the point of Figure 5 is to show that the data collapse to the same curve when everything is expressed in terms of free energy change, which I suppose is an important and interesting result. However, the figure is sort of a jumble. Is there a better way to show this?
18) Figure S1. Why is the SFP/CFP ratio different at t=0 than it is after serine is removed at t=5500?
19) Also Figure S1. What is being shown here? Are these trimers of receptor dimers? Are they supposed to be different homodimers or all of the same type? I am confused. I think the cartoon can be improved and explained more clearly.
20) Several figures. Why are standard errors rather than standard deviations shown?
21) What is the significance of the fact that the peak relative response to maltose is significantly lower in the ptsHI crr strain. Are the level of MBP lower? Were they quantified?
22) There is no reference to Figure S2B in the legend to the figure.
23) Figure S3 is very complicated. Maybe there is no way around that, but I am not sure that the pictures of flasks and tubes at the top are necessary or add much. Same with the flasks in Figure S4. Also, "standard" is misspelled in the last line of the figure legend.
24) Legend to Figure S5. The reference to Fig. 3C seems to be in error. Figure 3C is a histogram and has no points. Also, the reference to shades of gray does not seem to jibe with the figure, and error bar presumably "indicate" standard errors.
25) The gel shown in Figure S6 is of unacceptable quality.

Additional Correspondence 28 April 2010

Thank you very much for handling our manuscript and for your positive feedback. I hope I could ask you one question informally before we prepare the revised version: how important do you feel is the inclusion of the data on integration of multiple effector signals?

You write that "...experiments to investigate responses to complex mixtures of effectors as suggested by ref#2 (and similarly eluted to by ref#3) ... should significantly improve the general impact of the study and would thus be essential to complement the current results", and ref#3 indeed said "If the current study were extended to investigate or predict chemotactic response to complex mixtures of effectors, the impact of the current study would be greater".

As a matter of fact, we have such data already and they were part of the early version of the manuscript. However, we purposely excluded them from the submitted version to focus of the

manuscript on the difference in signalling between the two types of receptors and it's relation to the substrate uptake. And since ref#3 suggests that we should focus the manuscript even more in this direction, I am uncertain whether to put the data on signal integration back into the paper or not. We intended to publish them as a separate short manuscript on signal integration, but I wouldn't have a problem including them back here again. For me, it's rather a question of introducing an additional topic into the manuscript, which would indeed increase its scope but at the same time diffuse the focus.

I understand that the final recommendation on publication will be given by one of the referees, but you presumably have experience in such cases and I would highly appreciate your advice on that matter.

Additional Editorial Correspondence 28 April 2010

Thanks for your query. I am very happy to hear that you do have already data on chemotactic response to complex mixtures of effectors at hand. I would very much encourage incorporation of those, as ref#2 also states that without those the study would be of limited scope and thus more suitable for the rather more specialized literature.
I do see your point that this would increase the complexity of the matter. However, the numerous very constructive suggestions how to shape and focus the current message should enable to implement such a second part in a similar concise manner. As this has been requested from ref#2 as have been results on Aspartate from ref#3, their will be no way around appropriately addressing these concerns.

Please let me know if I can be of further assisstance!

Yours sincerely,

Editor
The EMBO Journal

1st Revision - Authors' Response 30 July 2010

Referee #1:

We thank Referee #1 for the very positive feedback and for appreciating the completeness and novelty of our study and the overall clarity of data presentation.

This report finds rather different values than a previous study by Li & Hazelbauer, which has been treated as gospel in the literature. I believe the new numbers, but suggest that the authors expend a few additional sentences to explain how they used fluorescently-tagged receptors to calibrate the western blots (if I understand the method used) and why their measurements differ from those of Li & Hazelbauer.

We apologize for a bit too concise description of the Western blot receptor quantification in the previous version of the manuscript. We have now added a more detailed description of the overall quantification procedure in the corresponding section of Results (page 9), particularly clarifying the usage of YFP as a neutral antigen to determine the antibody specificity for each receptor. We further slightly expanded the section on receptor quantification in Materials and methods, and illustrated the whole procedure step-by-step, including all calibration steps, in Supplementary Figs. S4 and S5 and in Supplementary methods. We also added a more detailed discussion of the differences between our study and the previous work by Li & Hazelbauer, and on the possible causes of these differences, in Results (pages 9-10) and in Discussion (page 17). We believe that one major source of discrepancy

is a strong dependence of relative receptor levels on the growth stage of the culture, which was not considered in that previous study, and have add a corresponding supplementary figure (Fig. S6) that demonstrates this dependence for minor receptors. Moreover, only the level of Trg was estimated directly by Li & Hazelbauer, whereas the estimation of Tap level was only indirect, which apparently led to a substantial underestimation.

I can only offer minor suggestions for improving clarity in spots.

1) p.2, lines 5-7: delete "directly", change "to demonstrate" to "and found" and delete comma after "new".

Changed according to suggestion.

2) p.3, line 4: change "is" to "are"

Changed.

3) p.3, line 6: delete "stable"

Deleted.

4) p.3, lines 11-12: add "to an attractant stimulus" before "works";

Changed to *'to a persistant attractant stimulus'*.

change "to attractants" to "the attractant".

Changed.

5) p.3, line 14: delete "clearly"

Done.

6) p.3, line 16: change "the first" to "one"; change "The second" to "a second".

Changed accordingly.

7) p.4, line 9: change "inside" to "within".

Changed according to suggestion.

8) p.4, line 21: add "the" before "corresponding"; line 23: change "is" to "are".

Changed accordingly.

9) p.5, lines 9-10: I wouldn't call these EC50 values "comparable". They seem to range over several logs.

We changed the phrasing to clarify that the natural ligands are sensed in a similar concentration range, whereas the non-metabolizable ligands are sensed less efficiently. Moreover, we specified the EC_{50} values for each ligand (page 6, second paragraph).

10) p.7, line 12: change "that" to "than".

Changed.

11) p.13, line 11: Perhaps add comma after "unsaturated".

Comma was added.

12) p.16, line 6: "microscope" is misspelled.

Spelling was corrected.

Referee #2:

We thank the referee for the critical comments and for suggestions on extending and improving our manuscript. We strongly believe that our study is of interest not only to those working on bacterial chemotaxis, but to a much broader community interested in optimal strategies of sensing and signalling and in bacterial behaviour in complex environments. Nevertheless, the Referee's suggestions are very valuable, and we have now added the requested experiments and expanded the discussion to further raise the general impact of our study.

If the current study were extended to investigate or predict chemotactic response to complex mixtures of effectors, the impact of the current study would be greater. Alternatively, a discussion of how the authors' observations impact bacteria in physiological settings or their ramifications for other signaling pathways also would raise the impact of the paper.

We followed the Referee's suggestion and included additional experiments on integration of chemotactic responses to multiple stimuli (new section of Results, pages 14-16, and new Fig. 6). These data clearly show that different stimuli are additive and that adaptation to a particular stimulus does not desensitize the system towards other ligands as long as there is not direct binding competition, which we argue represents the optimal strategy for gradient sensing in complex environments. We further expanded the discussion of the importance of our results for understanding bacterial behaviour in physiological settings.

General comments:

1. The manuscript is a very tough read in that is written bacterial chemotaxis insiders. It is difficult to discern the motivations behind the executed experiments. Context is missing also in that a discussion of reported prior experiments is needed.

We apologize if the motivation of our study was not presented clearly enough in the introductory section of our previous manuscript. We made changes to Introduction and Abstract, and have substantially modified the Discussion to further clarify the focus of the manuscript. Moreover, we modified the title to emphasize the main point of our study, as suggested by Referee #3. We did discuss our work in the context of previously published studies already in the previous version of our manuscript, but it was done in the Discussion section. We now refer to this previous work more clearly in the Introduction section, when defining the aims of our study. We believe that this has clarified both the context and motivation for our work.

2. While the FRET technique utilized here has been developed and used in previous reports, a brief overview of how the FRET experiments were conducted (in the main text) is needed so that readers can evaluate what the responses generated represent at a molecular level.

We added a more detailed explanation of the FRET assay at the beginning of Results. For better clarity and comprehensibility, we included an additional supplementary figure (Fig. S1) illustrating the assay and explaining relevant details.

a. The authors do not address in this manuscript whether the dynamic range of signaling observed is limited by the chemotaxis sensory system or by limits of the FRET system itself.

Any effects of ligands on the FRET readout are indirect and mediated through the kinase activity. Therefore, any potential limitations on the sensitive range of FRET are related to the range of measurable kinase activities, not to the applied range of ligand concentrations itself. We have previously shown that FRET depends linearly on the kinase activity within the entire physiological range of intracellular activities (this is mentioned now in Results), and the adapted kinase activity at high levels of ambient ligands always remains in this range.

b. How do the authors rule out that the signaling differences that they observe for the periplasmic binding proteins are not due to competing cellular uptake and metabolism of various signaling molecules? What is the affinity of the transporter versus the chemoreceptor for the BP-signal complex? How does BP uptake compare with internalization via symporters?

Since periplasmic binding proteins are believed to have much higher copy number than respective transporters (see comments of Referee #3), it is unlikely that the observed effects of BP titration are due to sequestration of BPs by the transporters. We have now also investigated the effects of ligand depletion due to uptake and metabolism on the concentration of individual attractants in our assay. We did observe a substantial reduction in the effective ligand concentration in the very low concentration range, which apparently led to an underestimation of the threshold sensitivity in our earlier dose-response experiments. To minimize the effects of reduction, we now performed dose-response measurements at much higher flow rate and corrected values in Fig. 1 and in Table SI. This effective reduction, however, was similar for directly and indirectly binding ligands. Moreover, it is only relevant for the very low range of tested ligand concentrations and is much smaller than the overall width of the dynamic range. It thus cannot explain the signalling differences between the two types of ligands. Nevertheless, we now discuss these issues in the text (pages 7 and 19), and we thank referee for pointing them out.

3. The authors quantified the amount of each receptor and used these amounts to normalize effector response. This analysis could be altered by processes noted in 2b above.

The ligand depletion does not affect receptor quantification, but it does affect the response in the low-concentration range and therefore the values of S_T, both non-normalized and normalized. As noted above, we now indicate corrected S_T values in Table SI. It has to be noted that in our study we do not draw any conclusions from the absolute values of S_T, since these values are primarily defined by the ligand affinity as much as by the signalling properties of receptors. Moreover, since the extent of depletion is very limited due to fast exchange of ligands in our assay, it neither affects the response sensitivity (S_R^P) nor the dynamic range at higher ligand concentrations – two parameters that characterize receptor behaviour in our analyses.

4. It was noted that the ratios of direct and indirect signaling receptors in this study differ from those previous reported, and this is a significant motivation for their proposed model in the Discussion. However, the authors did not cite those previous studies in the text. Moreover, what is lacking is an interpretation for the discrepancy in the quantity of receptors in this and previous studies. This ambiguity could lead readers to question which data is more reliable. Indeed, are the levels of receptors influenced by the growth conditions? If so, what levels are physiologically relevant?

We did cite the study by Li & Hazelbauer, 2004, in the Introduction of the previous version of our manuscript, but we did not discuss potential causes for the differences between the two studies. This is now corrected, and we also provide a more elaborate description of our quantification procedure (see our response to Referee #1). We identify the dependence of the relative expression levels of receptors on the cell culture density as one major cause of the observed differences, so both levels may be physiologically relevant. However, the main advance of our study was not just to determine receptor levels, but to correlate them with receptor responses observed under the same growth conditions.

5. The authors did not provide context to previous experiments that investigated the dynamic range of receptor response in the Results section. It appears that the values obtained in this study agree with those reported in the 1970s. It would be appropriate for the authors to discuss these previous experiments at the outset of the study. This would also provide more context to the importance of their findings with indirect binding ligands.

As mentioned above, we previously cited these experiments in the Discussion, but now also mention them in the Introduction (page 3).

6. It was unclear how the mathematical model augments this study. There was no context given in the Results section and little, if any, discussion provided. The authors should delineate what insights were gained from this updated model. Could they test their model with new experiments? Along

these lines, the data presented in Figure 5 were not evaluated. It was unclear what this data provided to the other findings described.

We elaborated on the importance of the model for the overall understanding of the differences in signalling between the two types of ligands in Results and in Discussion. The model indeed made some not-trivial predictions, for example for the dependence of the shape and peak position of the dynamic range on expression level of BP, which were later validated experimentally. We now mention these model-based predictions more clearly in the text (pages 12 and 19-20).

7. One of the main conclusions is that the smaller dynamic range of indirect ligand binding is not due to inefficient adaption, but due to the requirement for accessory binding to BPs. The authors do not provide sufficient experimental evidence to support their model.

We do think that both our data and mathematical analysis provide compelling evidence for this model. Fig. 2 and Fig. S3 clearly show that the dynamic range of the indirect ligand binding is not limited by adaptation, since cells stop responding to these ligands while still retaining high adapted activity. In contrast, the loss of response amplitude at high concentrations of directly binding ligands clearly correlates with the loss of adaptation. These differences are consistent with our detailed mathematical model of the two types of signalling.

8. Standard models for signaling output predict that processes that involve multiple proteins coming together will occur with a higher level of cooperativity and therefore one would predict periplasmic BPs should result in responses over a narrow range of concentrations.

Although such scenario is in principal possible, we disagree that involvement of multiple proteins must necessarily lead to higher cooperativity. Moreover, if the narrower dynamic range was a consequence of higher cooperativity (e.g., due to cooperative BP binding), one would have also expected higher response sensitivity at the peak, which was not observed.

8. Minor Comments. Several issues are unclear in the text as noted below.

a. Figure 4C - is the WT panel mislabeled, or is the description provided incorrect? If the description is correct, the WT cells should provide the lowest response to effector.

We apologize for this being unclear. The panel labelling and description are correct, but the WT cells in Fig. 4C indeed show a response that matches that of the highest induction level of GBP, which supports our conclusion that the galactose binding protein is fully induced in our wild type strain. This is now clarified in the respective Results section on page 11.

b. The last sentence on page 11 in the discussion was unclear. Most of this seems to be due to imprecise wording. It is unclear what the authors mean by "non-optimal binding properties of the BP" in regards to galactose signaling.

We rephrased the sentence and elaborated on possible reasons for the lower response sensitivity for indirectly binding ligands (page 17-18).

c. The sentence on page 12 referring to Koff and Kon is confusing. Are the authors referring to rate constants (typically lower case k) or are these equilibrium constants for the on and off states? If it is the latter, the nomenclature is not parallel to that used earlier.

This is now corrected by using the same nomenclature throughout the text. We apologize for this confusion.

d. The use of the term "saturation" with regard to the receptors is confusing. Saturation with receptor ligand interactions has a very different meaning so I found the use of "saturating" levels of receptor to be confusing.

The sentence was rephrased.

Referee #3:

This is a monumental study, impressive for its thoroughness both in experiment and modeling. It has one major drawback, however, which is that it is relatively hard to read. I feel that the authors have failed to make the best case that they can for the importance of their results. The paper should be rewritten to increase its clarity and accessibility.

First of all, we would like to thank the referee for recognizing the scope and thoroughness of our work, and for extremely helpful comments for manuscript improvement. We have now substantially rewritten the manuscript to improve its clarity and accessibility. We modified the title and extended the discussion to emphasise the key findings of our work, and to better explain the importance of our observations for understanding coordination between chemotaxis and nutrient uptake in bacteria.

Transport does, however, significantly diminish the level of substrate available in the periplasm at moderate concentrations of maltose – and presumably other binding-protein substrates - which has the effect of broadening the dynamic range for chemotaxis and shifting it toward higher concentrations. For example, cells that are defective for maltose transport show peak accumulations in capillary assays at tenfold lower concentrations of maltose than cells with intact transport systems (see Zhang et al., JBC 1999, and references therein).

We thank the referee for pointing it out. As elaborated in the response to question 2b of Referee #2, we have investigated the effects of ligand depletion due to uptake and metabolism on the concentration of individual attractants in our assay and did observe a substantial effect in the very low concentration range (Fig. S2 and Table SI). To minimize the effects of ligand depletion on the threshold sensitivity, we now measured dose responses in Fig. 1 at much higher rate of ligand exchange in the flow chamber, despite adverse effects of such high flow rate on the overall quality of our measurements. This observed ligand depletion, however, was similar for directly and indirectly binding ligands, and was limited to lowest ligand concentrations, and therefore does not affect our conclusions about differences between the two types of ligands. Neither should it affect the values of response sensitivity, which were measured at much higher ligand concentrations. We now discuss this point in the text (pages 7 and 19). We further performed control experiments with mutants deleted for transporters of maltose and galactose. For maltose, we indeed observed a shift in the dynamic range and in the dose-response curve to lower ligand concentration, in agreement with Zhang et al., JBC 1999 and with our results on ligand depletion. However, for galactose the shift was in the opposite direction, suggesting that transporter deletion can have other effects, for example changing expression of binding protein. We therefore did not show these results, but we did cite Zhang et al.

Some of these points are made in the Discussion of the current manuscript, but almost as an afterthought. I think they should be front and center. A title that would embody this idea would be something like "Correlation of mechanisms for chemotaxis signaling and substrate transport in Escherichia coli." If the authors insist on focusing on chemoreceptor function, they should still emphasize the difference, as in a title like "Chemoreceptors signal differently in response to ligands that bind directly and indirectly." In any case, the Abstract should be rewritten to highlight the really interesting, rather than the more arcane, inferences from the work.

We changed the title to “Differences in signalling by directly and indirectly binding ligands in chemotaxis of *Escherichia coli*“ and modified the Abstract.

The main question that I have about the experimental results is why aspartate was never tested. It is the direct-binding attractant with the lowest EC50. It would seem to be important to include aspartate to make the comparison complete, since serine and its non-metabolizable, chemotactically less-potent analog AIBU were both tested. I realize that, at high concentrations, it may also be sensed by Tsr, but that should interfere too much with the measurements at micromolar concentrations.

Data for aspartate were added to the manuscript. This did not change any of our conclusions, but indeed helped to make the point that natural (= metabolizable) ligands of both types are sensed with similar threshold sensitivity, presumably pointing at an evolutionary optimization up to a physical limit of sensitivity. We thank the referee for this suggestion.

That brings up one other concern, which is the invocation of a mysterious binding protein that could mediate a Tsr-independent response to serine. Why couldn't that just be a low-level response to serine mediated by Tar? This hypothetical binding protein, whose existence is based on circular logic, i.e., a marginally smaller dynamic range, should be eliminated unless there is firmer evidence for its existence.

This is a justified objection and we deleted the speculative assumption of a BP for serine. This response may be indeed mediated by Tar, since in our additional experiments performed for this revision it was observed in a Tar-only strain. However, serine showed a much narrower dynamic range than that of other tested ligands of Tar, so we still don't know whether the response is mediated by direct serine binding to Tar or by some transport-related mechanism that requires Tar for kinase activation. We mention it now accordingly in the text on page 10.

I have a number of minor comments, in addition to many suggestions for changes in wording and punctuation, which can wait for a later time:

1) Citations clustered in the text should be in chronological order.

Citations were put into chronological order.

2) Macnab and Koshland should also be given credit to temporal comparisons of chemoeffector concentrations.

The reference is included.

3) Hazelbauer (1975) was the first to show that MBP is the primary sensor of maltose, and Manson and Kossmann (JB, 1986), not Manson et al. (1985) were the first to give solid genetic evidence for the direct interaction of MBP and Tar.

We changed the citations accordingly.

4) On the first page of Results, the statement that all ligands showed comparable EC50 values is very misleading and in direct contradiction to the date shown in Figure 1.

We changed the phrasing to clarify that the natural ligands are sensed in a similar concentration range, whereas the non-metabolizable ligands are sensed less efficiently. Moreover, we specified the EC_{50} values for each ligand in the text (page 6, second paragraph).

5) On the same page, it should be briefly explained here in what strains and how binding protein were overexpressed.

We now mention the effects of BP overexpression on S_T at the end of the section on Response sensitivity on page 11, and explain in this section how BPs were overexpressed. It is also explained in the legend of Fig. 3.

6) Is there a PTS for maltose? Isn't the response likely to be to glucose contamination? Otherwise, why would cells lacking the ABC transporter for maltose be unable to grow on even high concentrations of pure maltose?

We thank the referee for this suggestion. We underestimated the extent of the contamination of even the highest-grade commercial maltose by glucose. Our additional experiments performed for maltose in a *trg* mutant revealed that the second peak in the dynamic range of maltose is indeed due to glucose contamination. We therefore removed data points corresponding to the high range of maltose concentrations in our dynamic range measurements; the same was done for galactose. We added a section '*Attractants'* to Materials and methods to specify the purity grade of chemicals, where we point out that glucose contamination can lead to an unspecific response at high concentrations of these two ligands.

7) Page three of results. It should be explained briefly in the text how the immunoblots were quantified. Also, in the quantification of chemoreceptors in strains W3110 and RP437 (Fig. S3D and

E) really comparable, and even if so, how do the authors explain the very different results of Li and Hazelbauer (2004)?

We have now described receptor quantification in much more detail and added a likely explanation of the differences between our study and the work by Li & Hazelbauer (see our response to Referee #1 for details).

8) Page four of Results. No data are given for Tar in Figure 5SD, as the text implies.

The sentence was rephrased.

9) Same page, what is "the total receptor level?" Is this relative to TSR with a value of 1? Not clear.

"The total receptor level" indeed refers to the native level of Tsr. This is now clarified.

10) Top of next page. When the authors say the Kon and Koff are binding constants for a particular type of receptor, does that mean a particular receptor in a particular methylation state? Because methylation can change ligand affinities (an unexplored topic for indirectly binding ligands, as far as I know), it is important to be very clear about this.

All current model of allosteric receptor signalling, including ours, assume that even for directly binding ligands Kon and Koff only depend on receptor activity and not on receptor methylation per se. Methylation does affect the affinity in the model, as suggested by experiments, but this effect is mediated by the effect of methylation on activity. The same assumption is made here for indirectly binding ligands.

11) First page of Discussion, bottom line. What is meant by "the non-optimal binding properties of the BP?"

We rephrased the sentence and extended the paragraph to further discuss possible reasons for the differences in response sensitivity to different ligands (page 17-18).

12) Break up the run-on paragraphs in the Discussion into more digestible segments.

Done.

13) It seems odd to say (see text for details) in the Materials and Methods.

Sentence was changed accordingly.

14) Bottom of the first page of Material and Methods, the sentence beginning here and continuing to the next page is long and tortuous.

The sentence was simplified.

15) Second page of Material and Methods. Is "480/40 nm band pass" correct, and if so, what does it mean? ± 40 nm, 460-500 nm?

It means that the center wavelength of the filter is 480nm and the full bandwidth is 40nm, e.g. 480 +/- 20nm. It is a standard notation used in the literature, so we did not change it.

16) Could the reason that the titration curves with galactose, ribose and Pro-Leu do not approach zero kinase activity be that some receptor teams do not contain Trg or Tap. (That would seem more likely with Li and Hazelbauer's receptor ratios.)

Not necessarily, since having just one receptor of a particular kind in a team may not be enough to fully inhibit team activity. This depends on binding parameters of ligand and/or of binding protein, and on the expression level of binding proteins. Consistent with that, we clearly observe that response amplitudes to saturating stimuli increase with the level of BP expression, while receptor levels stay unchanged. Moreover, we can well reproduce the data with the model that uses relative

receptor abundances determined in our study, suggesting that our experimental and modelling results are self-consistent.

17) I realize that the point of Figure 5 is to show that the data collapse to the same curve when everything is expressed in terms of free energy change, which I suppose is an important and interesting result. However, the figure is sort of a jumble. Is there a better way to show this?

We now emphasize more the significance of the model for our study, see our response to question 6 of Referee #2. Energy collapse with all curves falling on one line is an important mean to demonstrate the validity of the model assumption that all response features can be explained by the free-energy dependence of the receptor activity.

18) Figure S1. Why is the SFP/CFP ratio different at t=0 than it is after serine is removed at t=5500?

The increase of the YFP/CFP ratio results from gradual photobleaching of the FRET donor CFP that is constantly excited during the measurement. We mentioned it in the figure legend. Moreover, we now added a supplementary figure (Fig. S1) to better explain the FRET assay, which also illustrates the bleaching effect.

19) Also Figure S1. What is being shown here? Are these trimers of receptor dimers? Are they supposed to be different homodimers or all of the same type? I am confused. I think the cartoon can be improved and explained more clearly.

We apologize for the lack of clarity of the previous figure legend. We now expanded the legend for this figure (now Fig. S3) to provide a more clear interpretation of the symbols and colours used.

20) Several figures. Why are standard errors rather than standard deviations shown?

Standard errors define precision of the determined mean and are more suitable here than standard deviations which illustrate variability in the measurements.

21) What is the significance of the fact that the peak relative response to maltose is significantly lower in the ptsHI crr strain. Are the level of MBP lower? Were they quantified?

The question is no longer relevant, since the response was due to glucose contamination [see (6)]. The figure was removed.

22) There is no reference to Figure S2B in the legend to the figure.

We apologize and thank the referee for noticing that. A reference to this figure (now Fig. S7) was added (page 10).

23) Figure S3 is very complicated. Maybe there is no way around that, but I am not sure that the pictures of flasks and tubes at the top are necessary or add much. Same with the flasks in Figure S4. Also, "standard" is misspelled in the last line of the figure legend.

The flasks and tubes in both figures (now Fig. S4 and Fig. S5) were removed to simplify the figure, and the figure legend was further improved to clarify the procedure. The spelling of “standard” was corrected.

24) Legend to Figure S5. The reference to Fig. 3C seems to be in error. Figure 3C is a histogram and has no points. Also, the reference to shades of gray does not seem to jibe with the figure, and error bar presumably "indicate" standard errors.

The reference in the figure legend of former Fig. S5 (now Fig. S8) was corrected. The reference to gray shading was removed. Explanation of error bars was included.

25) The gel shown in Figure S6 is of unacceptable quality.

The experiment was repeated and a blot of much improved quality is now shown in Fig. S9 A. To facilitate reading, we further added a profile of receptor distribution to the figure.

2nd Editorial Decision 16 August 2010

I just received the final assessments from two of the original referees. You will be pleased to learn that on the basis of this you will soon receive the official acceptance letter together with further instructions from our editorial assistance.

I assume that the citation order could be sorted out during the proof stage!

With my best regards.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Ref#2:

It was a pleasure to read this revised manuscript, which provides new insight into the process of chemotactic signaling. In addition to the data and the model presented, the classification of receptors as direct and indirect will be valuable the field.

The authors have responded to the reviewer criticisms effectively. Most significantly, the clarity of presentation has been enhanced dramatically. With this presentation, I concur with the authors that their results are of broad significance. Additionally, all of my scientific concerns have been addressed.

Ref#3:

This was a very promising manuscript in its original form. That promise has now been fulfilled. The responses to the reviewers comments were constructive and have improved the manuscript substantially. I think that this paper sets a standard for excellence that should be emulated by everyone working in the field.

Oh, by the way, on line 2 of page 19, the two citations are in the wrong chronological order. For shame!

